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The virus-host interactions between Japanese encephalitis (JE) virus and mouse brain neurons were analyzed by electron microscopy. JE virus replicated exclusively in the rough endoplasmic reticulum (RER) of neurons. In the early phase of infection, the perikaryon of infected neurons had relatively normal-looking lamellar RER whose cisternae showed focal dilations containing progeny virions and characteristic endoplasmic reticulum (ER) vesicles. The reticular RER, consisted of rows of ribosomes surrounding irregular-shaped, membrane-unbounded custernae and resembled that observed in JE-virus-infected PC12 cells, were also seen adjacent to the lamellar RER. The appearance of the reticular RER indicated that RER morphogenesis occurred in infected neurons in association with the viral replication. The fine network of Golgi apparatus was extensively obliterated by fragmentation and dissolution of the Golgi membranes and their replacement by the electron-lucent material. As the infection progressed, the lamellar RER was increasingly replaced by hypertrophic RER which had diffusely dilated cisternae containing multiple progeny virions and ER vesicles. The Golgi apparatus, at this stage, was seen as coarse, localized Golgi complexes near the hypertrophic RER. In the late phase of

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infection, RER of infected neurons showed a degenerative change, with the cystically dilated cisternae being filled with ER vesicles and virions. Small, localized Golgi complexes frequently showed vesiculation, vacuolation, and dispersion. The present study, therefore, indicated that during the viral replication the normal lamellar RER which synthesized neuronal secretory and membrane proteins was replaced by the hypertrophic RER which synthesized the viral proteins. The hypertrophic RER eventually degenerated into cystic RER whose cisternae were filled with viral products. The constant degenerative change which occurred in the Golgi apparatus during the viral replication suggested that some of the viral proteins transported from RER to the Golgi apparatus were harmful to the Golgi apparatus and that increasing damage to the Golgi apparatus during the viral replication played the principal role in the pathogenesis of JE-virus-infected neurons in the central nervous system.

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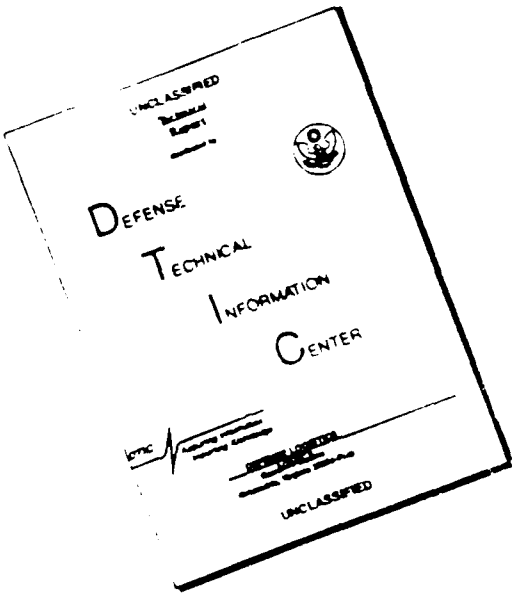

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Virus-neuron interactions in the mouse brain infected with Japanese encephalitis virus

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Summary. The virus-host interactions between Japanese encephalitis (JE) virus and mouse brain neurons were analyzed by electron microscopy. JE virus replicated exclusively in the rough endoplasmic reticulum (RER) of neurons. In the early phase of infection, the perikaryon of infected neurons had relatively normal-looking lamellar RER whose cisternae showed focal dilations containing progeny virions and characteristic endoplasmic reticulum (ER) vesicles. The reticular RER, consisted of rows of ribosomes surrounding irregular-shaped, membrane-unbounded cisternae and resembled that observed in JE-virus-infected PC12 cells, were also seen adjacent to the lamellar RER. The appearance of the reticular RER indicated that RER morphogenesis occurred in infected neurons in association with the viral replication. The fine network of Golgi apparatus was extensively obliterated by fragmentation and dissolution of the Golgi membranes and their replacement by the electron-lucent material. As the infection progressed, the lamellar RER was increasingly replaced by the hypertrophic RER which had diffusely dilated cisternae containing multiple progeny virions and ER vesicles. The Golgi apparatus, at this stage, was seen as coarse, localized Golgi complexes near the hypertrophic RER. In the later phase of infection, RER of infected neurons showed a degenerative change, with the cystically dilated cisternae being filled with ER vesicles and virions. Small, localized Golgi complexes frequently showed vesiculation, vacuolation, and dispersion. The present study, therefore, indicated that during the viral replication the normal lamellar RER which synthesized neuronal secretory and membrane proteins was replaced by the hypertrophic RER which synthesized the viral proteins. The hypertrophic RER eventually degenerated into cystic RER whose cisternae were filled with viral products. The constant degenerative change which occurred in the Golgi apparatus during the viral replication suggested that some of the viral proteins transported from RER to the Golgi apparatus were harmful to the Golgi apparatus and that increasing damage to the Golgi apparatus during the viral replication played the principal role in the pathogenesis of JE-virus-infected neurons in the central nervous system.

Key words: Golgi apparatus – Japanese encephalitis virus – Mouse brain neuron – Rough endoplasmic reticulum – Viral infection

Introduction

The viral genomic RNA (vgRNA) of JE virus forms the vgRNA-polysome that functions exclusively at the RER of the host cell for the synthesis of the viral proteins (Hase et al. 1987, 1989, 1990a). The viral proteins synthesized on the vgRNA-polysome at RER are discharged into the RER cisterna for the assembly of progeny virions; the assembled virions are then transported through the host secretory channel via the Golgi apparatus to be released extracellularly by secretory-type exocytosis. In this respect, the vgRNA of JE virus behaves like cellular messenger RNA (mRNA) for secretory and membrane proteins, and progeny virions assembled in the cisternae behave like host secretory material by passing through the host secretory channel for extracellular release. It is apparent, therefore, that JE virus replication depends on the host secretory activity.

It has been shown recently that JE virus infection of a cultured neurogenic cell line, PC12 cells, causes massive proliferation of RER in the host cytoplasm in association with the viral replication (Hase et al. 1992; Hase 1993). Although JE virus infection of mouse brain neurons does not seem to cause such massive RER proliferation as that of cultured PC12 cells, it has been shown that infected neurons display the hypertrophic RER whose diffusely dilated cisternae contain multiple virions and ER vesicles (Hase et al. 1987, 1990a). Therefore, in the light of massive RER morphogenesis that occurs in JE-virus-infected PC12 cells, a question arises as to whether the hypertrophic RER in infected neurons involves only hyper-

trophic change of the existing RER or it involves formation of new RER. It is of great interest to see whether or not an exogenous mRNA such as the vgRNA of JE virus, introduced directly into the cytoplasm, induces morphogenesis of the cellular secretory organelles in tissue cells. Accordingly, in this study the ultrastructural changes of the protein secretory system including RER and the Golgi apparatus in JE-virus-infected neurons of the mouse brain are re-examined to see whether morphogenesis of these secretory organelles are involved during the viral replication.

Materials and methods

The Chinese strain SA14 of JE virus was used. The purified virus was prepared according to the procedure described previously (Hase et al. 1987). The procedures for infecting mice with JE virus and preparing brain tissues for electron microscopical examination were described in detail elsewhere (Hase et al. 1990a). Briefly, mice (ICR strain, about 4 weeks old) were inoculated intracerebrally with 0.03 ml (10^7 PFU) of the purified virus, and two or more mice were sacrificed daily from day 3 to day 6 postinoculation (p.i.). For the control, two mice inoculated intracerebrally with the same amount of virus-free diluent (Eagle's minimum essential medium) were

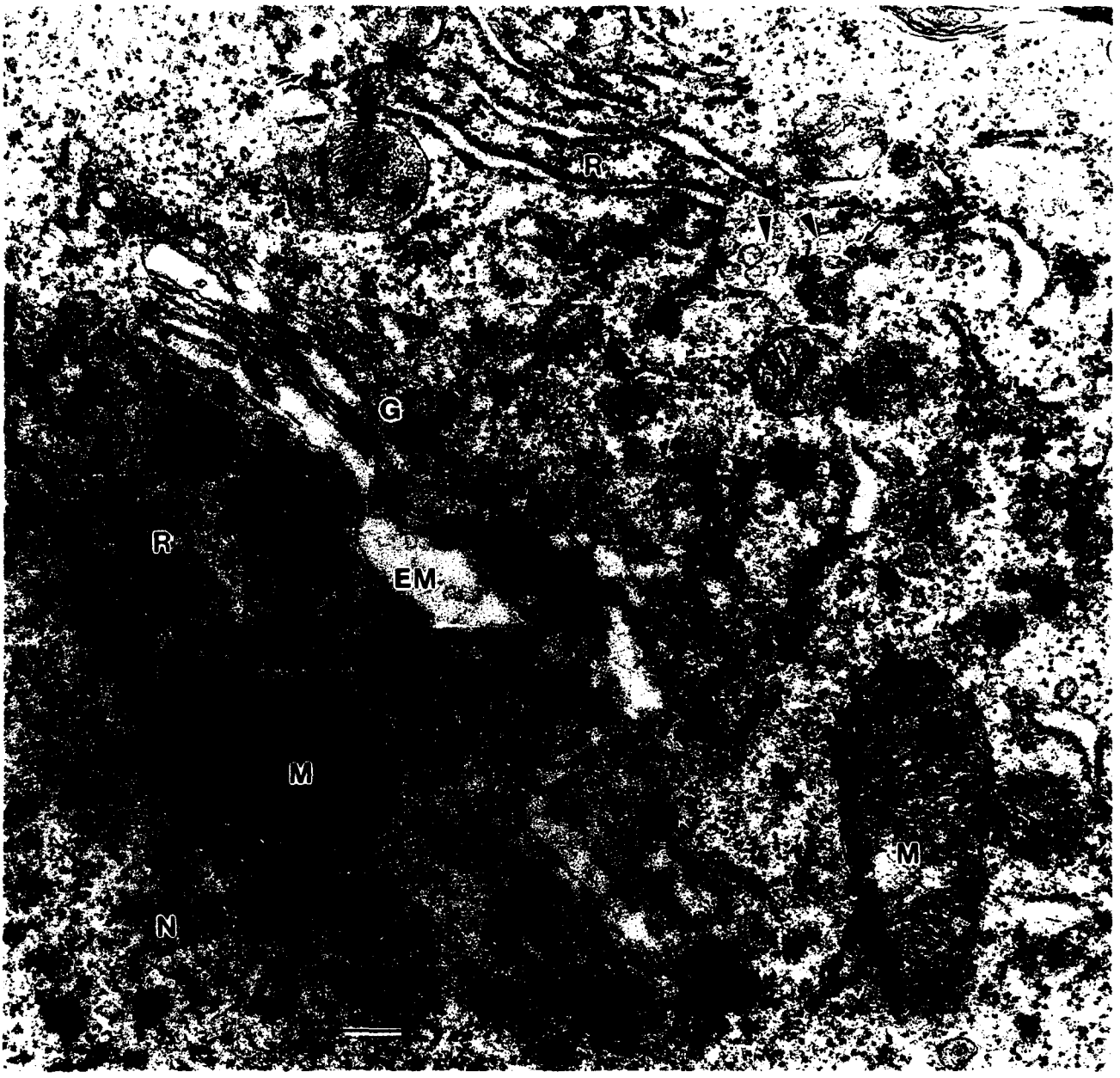

Fig. 1. A JE-virus-infected neuron in the cerebral cortex, 3 days postinoculation (p.i.). The perikaryon displays normal-looking lamellar RER (*R*); it also shows reticular RER consisted of rows of ribosomes surrounding membrane-unbounded, irregular-shaped cisternae *(thick arrow)*. Virions *(small arrow)* and characteristic endoplasmic reticulum (ER) vesicles (arrowhead) are seen in the RER cisternae. The fine membrane network of the Golgi apparatus (*G*) is obliterated and replaced by electron-lucent material (*EM*) in places. *L*, lysosome; *M*, mitochondrion; *N*, nucleus. ×45000; *bar* = 200 nm

sacrificed at each period. The mice were anesthetized and perfused first with 20 ml of cold phosphate-buffered saline, followed by 20 ml of cold 4F1G (McDowell and Trump 1976), by injecting the fluids from the left ventricle under a mild pressure and draining them from a cut in the right atrium. The perfused animals were placed in a refrigerator at 4° C for 30 min. The brains were excised, and tissue samples measuring about 1 mm^3 were taken from the cerebral cortex, the basal ganglia-thalamic region, the cerebellar cortex, and the medulla oblongata. The tissue samples were fixed in 4F1G fixative at 4° C overnight, washed in 0.1 M cacodylate buffer (pH 7.4), postfixed in 1% cacodylate-buffered osmium tetroxide, dehydrated, and embedded in Epon 812. Thin sections were cut on a LKB Ultrotome, Nova, placed on uncoated grids, stained with uranyl acetate and lead citrate, and examined with a Zeiss SM109 electron microscope.

Results

In the early phase of infection, progeny virions and ER vesicles were found in the cisternae of relatively normal-looking lamellar RER of infected neurons (Fig. 1, 2). This apparently indicated that the vgRNA-polysomes initially joined to the existing RER for the synthesis of the viral proteins. Nonetheless, the cisternal sites of lamellar RER that contained virions and ER vesicles often showed irregular dilations and partial obliteration of limiting RER membranes (Fig. 2). A structure, that consisted of rows of ribosomes surrounding irregular-shaped, membrane-unbounded cisternae and closely re-

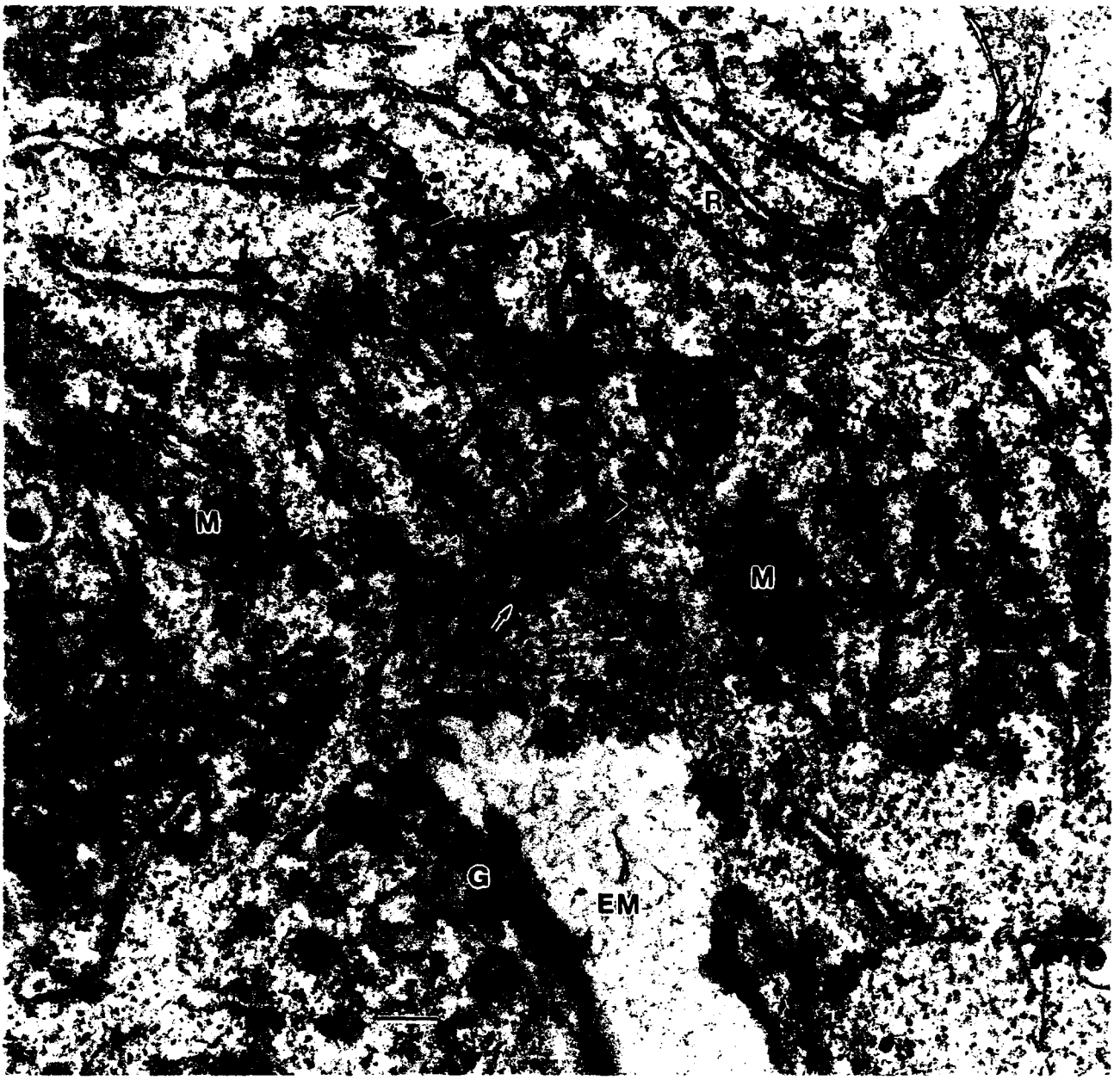

Fig. 2. The perikaryon of a JE-virus-infected neuron in the cerebral cortex, 3 days p.i. The lamellar RER (*R*) contains areas where focal dilations of cisternae containing virions *(arrow)* and ER vesicles *(arrowhead)* are present. The dilated cisternae frequently lack limiting RER membranes. The Golgi apparatus (*G*) in the picture is almost completely replaced by the electron-lucent material (*EM*). *M*, mitochondrion. × 45000; *bar* = 200 nm

sembled the reticular RER observed in JE-virus-infected PC12 cells (Hase 1993), were seen adjacent to the lamellar RER (Fig. 1). Like that in JE-virus-infected PC12 cells, the structure was thought to represent nascent RER and was called the reticular RER. Virions and ER vesicles were frequently found in the membrane-unbounded cisternae of the reticular RER (Fig. 1). By far, the most pronounced morphological change in the early phase was observed in the Golgi apparatus whose delicate membrane network was extensively obliterated by membrane fragmentation and dissolution and focal or diffuse replacement of the membrane structure by characteristic amorphous, electron-lucent material (Fig. 1–3). The electron-lucent material seemed to initially accumulate focally, distending Golgi saccules and fragmenting the Golgi membranes (Fig. 1, 3). Eventually, however, it obliterated almost the entire Golgi structure in places (Fig. 2). Vesicles, some of which containing virions or ER vesicles, were seen in the Golgi region, indicating that the viral products manufactured in RER were actively transported to the Golgi apparatus (Fig. 3). These vesicles apparently fused to the Golgi apparatus, delivering their contents including virions into the Golgi saccules. The virions appeared in the Golgi saccules, but the ER vesicles did not appear in the Golgi saccules and seemed to dissolve before or at the time when the vesicles carrying them fused to the Golgi membrane, presumably delivering their content into the Golgi saccules.

As the infection progressed, many infected neurons displayed in their perikaryon the hypertrophic RER that had diffusely dilated cisternae in which multiple virions and ER vesicles were found (Fig. 4, 5). The hypertrophic RER occurred either as randomly scattered, short segments or as broad, branching plates in certain areas of the perikaryon. Admittedly, the membrane continuity of a membrane-limited structure is difficult to confirm in electron microscopy because of the variations that occur in the membrane image depending on the cutting angle. Nonetheless, large areas of the cisternal surfaces of the hypertrophic RER lacked the limiting RER membranes

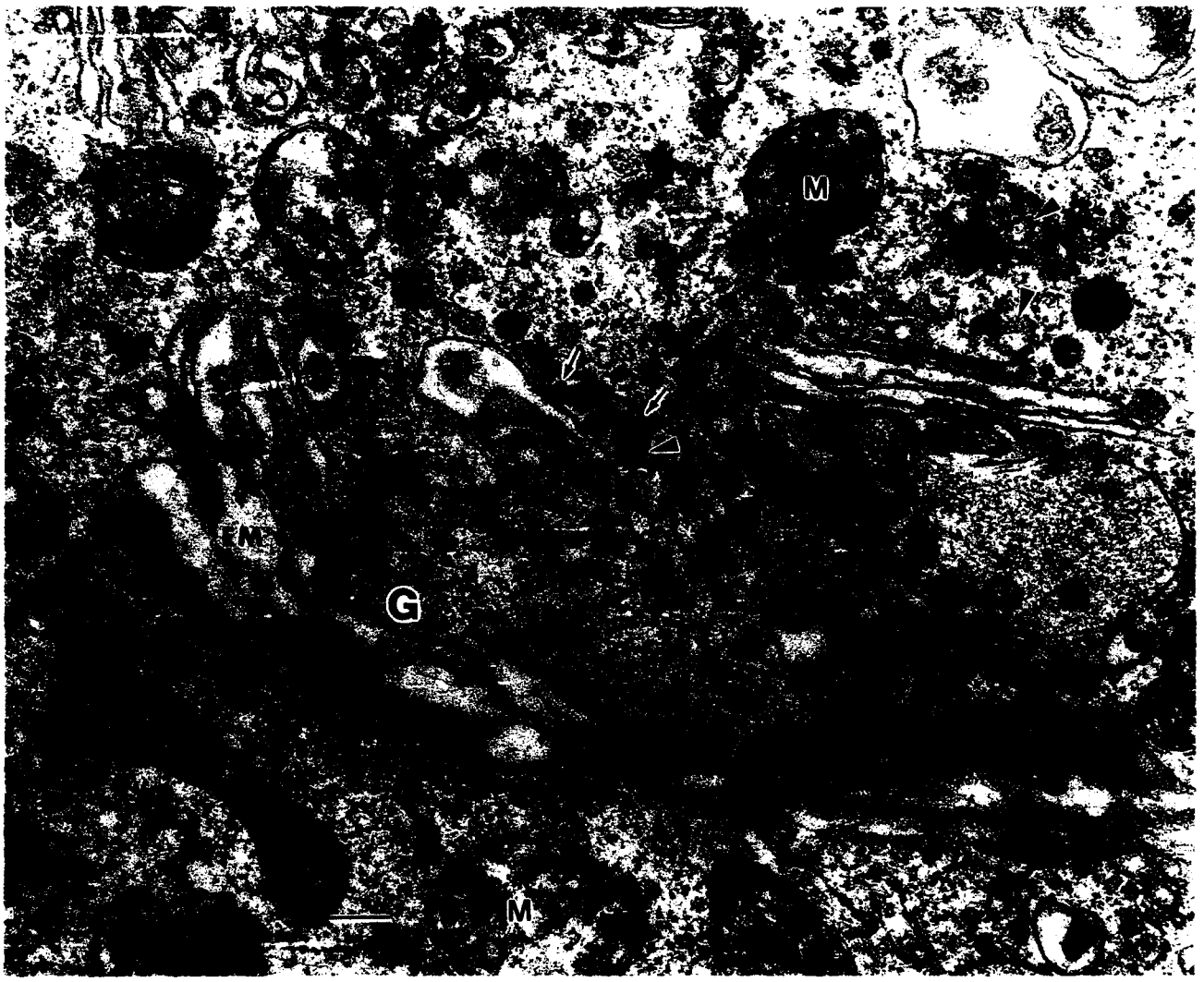

Fig. 3. The Golgi apparatus *(G)* of a JE-virus-infected neuron in the cerebral cortex, 3 days p.i., showing fragmentation and dissolution of Golgi membranes and their replacement by electron-lucent material *(EM)*. Note the presence of membrane vesicles carrying virions *(arrow)* and ER vesicles *(arrowhead)* in the Golgi region. *M*, mitochondrion, ×45000; *bar* = 200 nm

and, instead, were bordered by the zones of mildly electron-dense, fuzzy material in which rows of ribosomes were embedded (Fig. 4, 5). Fine stringy projections of mildly electron-dense material extended from ribosomes embedded in the fuzzy zones into the cisternal lumen, frequently connecting with virions within the cisternae; these projections might represent some kinds of proteins synthesized on the ribosomes and extending into the cisternal lumen. In the dilated cisternae of the hypertrophic RER, roughly three forms of progeny virions were recognized: (a) the dense form which showed an electron-dense nucleocapsid tightly wrapped around by a membrane envelope (1 in Fig. 5), (b) the bull's-eye form which showed a small, electron-dense nucleocapsid core clearly separated from a membrane envelope by an electron-lucent space (2 in Fig. 5), and (c) the light form in which a virus particle appeared as a mildly to moderately electron-dense, homogeneous particle without internal structural differentiation (3 in Fig. 5). The dense form was also seen extracellularly and apparently represented the mature form. On the other hand, whether the other two forms represented virus particles at different maturation stages or certain defective forms could not be determined in this study. As shown later, many virus particles that were found in cistically dilated cisternae of the degenerating RER in the later phase of infection showed the bull's-eye form. The fine network of Golgi apparatus seen in normal neurons disappeared completely from infected neurons and replaced by one or more coarse, localized Golgi complexes (Fig. 6). Membrane vesicles

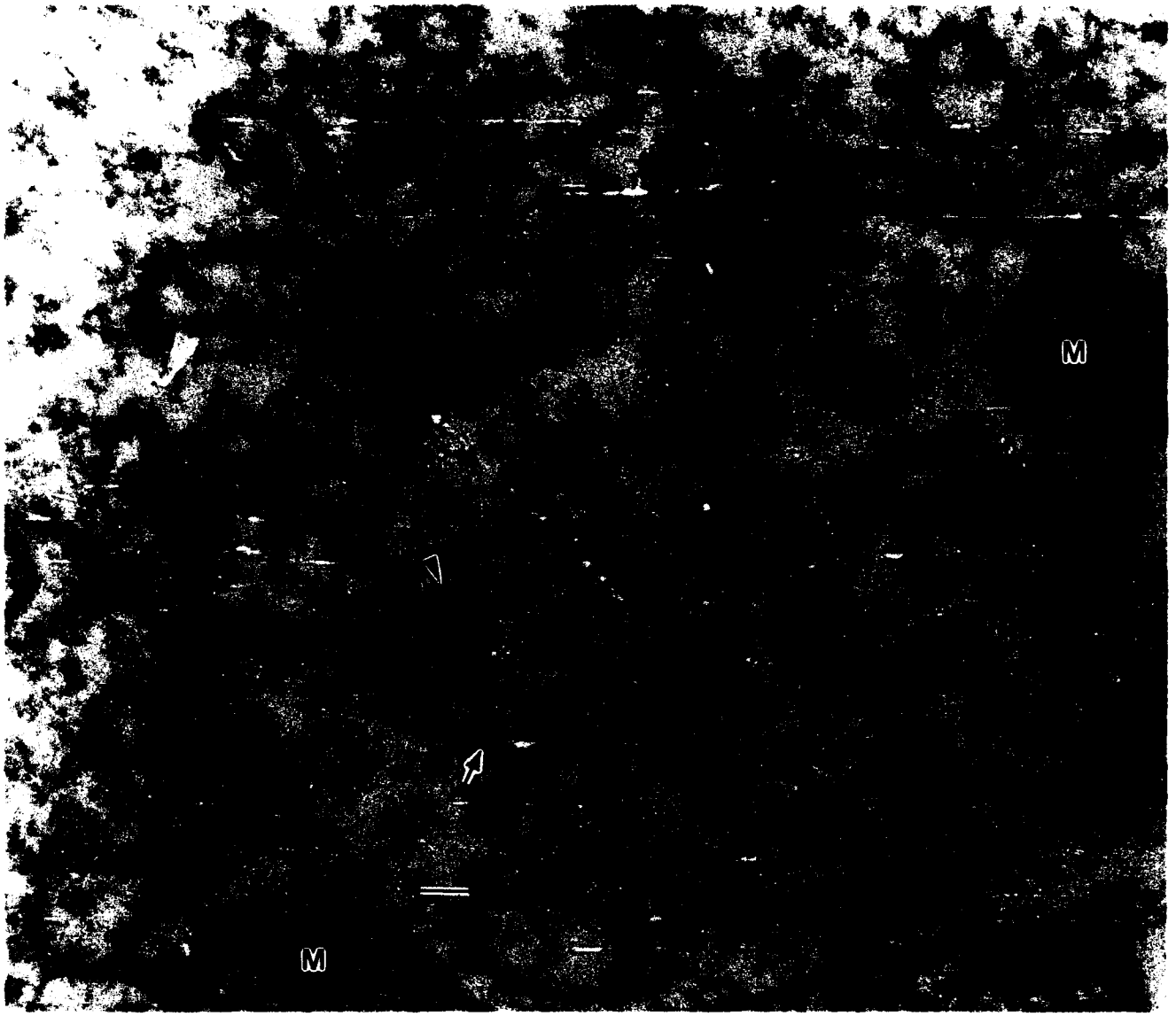

Fig. 4. Irregularly scattered, short segments of hypertrophic RER in the perikaryon of a JE virus-infected neuron, 4 days p.i. The RER cisternae are dilated and contain virions *(arrow)* and ER vesicles *(arrowhead)*. Note that large areas of cisternal surfaces lack limiting RER membranes and, instead, are bordered by zones of mildly electron-dense fuzzy material in which rows of ribosomes are embedded. M, mitochondrion, × 72000; *bar* = 100 nm

carrying virions or ER vesicles were seen around the Golgi complexes. These vesicles apparently fused to the Golgi complexes. Virions were also seen within coated vesicles in the Golgi region, indicating that the Golgi apparatus released virions into the cytoplasm within coated vesicles for secretion. The Golgi complexes continued to show focal membrane dissolution and accumulation of the electron-lucent material (Fig. 6).

In the later phase of infection, the hypertrophic RER becamc increasingly degenerative, with segmentation and cystic dilation of its cinsternae (Fig. 7). The cisternae of the cyctic RER were mostly delineated by limiting RER membranes whose cytosolic surfaces were sparsely lined by ribosomes. The cystically dilated cisternae were usually packed with ER vesicles and virions. Many virus particles in the cystically dilated cisternae showed the bull's-eye form (Fig. 7). The Golgi apparatus was not prominent and scattered as small localized Golgi complexes (Fig. 8). Golgi complexes at this stage frequently showed vesiculation, vacuolation, and dispersion. Nonetheless, virions were seen in the saccules of these degenerating Golgi complexes and within coated vesicles

Fig. 5. Hypertrophic RER showing a branching pattern in the perikaryon of a JE-virus-infected neuron, 4 days p.i. The dilated cisternae contain virions *(arrow)* and ER vesicles *(arrowhead)*. Large areas of cisternal surfaces lack RER membranes and are bordered by the fuzzy zones in which rows of ribosomes are embedded. Note the fuzzy strings of mildly electron-dense material that project from the ribosomes into the cisternal lumen. 1, the dense form; 2, the bull's-eye form; 3, the light form; M, mitochondrion. 96000; *bar* = 100 nm

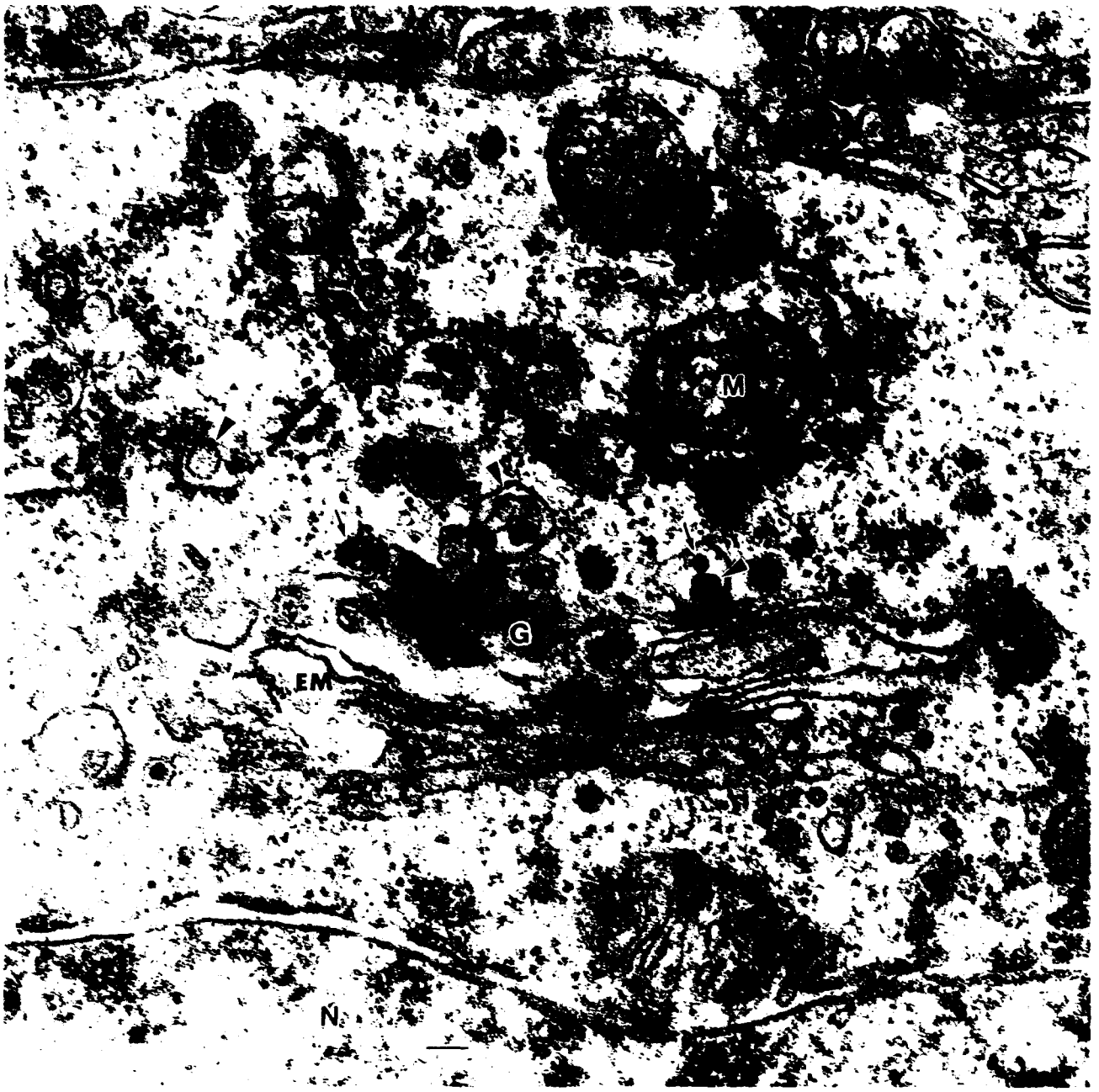

Fig. 6. A JE-virus-infected neuron in the cerebral cortex. 5 days p.i. RER is fragmented and disoriented. The irregularly dilated RER cisternae are bordered by rows of ribosomes without clearly recognizable RER membranes and contain virions *(arrow)* and ER vesicles *(arrowhead)*. The saccules of the Golgi apparatus (*G*) show focal dilations containing the electron-lucent material (*EM*). Note the presence of membrane vesicles carrying virions *(arrow)* and ER vesicles *(arrowhead)* in the Golgi region. *M*, mitochondrion; N, nucleus. × 66000; *bar* = 100 nm

in the Golgi region, indicating that the Golgi complexes were still processing virions and releasing them into the cytoplasm within coated vesicles for secretion.

Discussion

The present study demonstrated that during JE virus replication the lamellar RER of normal neurons was progressively altered to the characteristic hypertrophic RER whose dilated cisternae contained multiple virions and ER vesicles. The change seemed to take place as the cellular mRNA-polysomes, which normally associate with the lamellar RER and on which neuronal secretory and membrane proteins are synthesized, were increasingly replaced by the vgRNA-polysomes on which the viral proteins were synthesized during the viral replication. At the same time, the appearance of the reticular RER suggested that RER morphogenesis in JE-virus-infected neurons occurred in the same manner as that in JE-virus-

Fig. 7. Cystically degenerated RER in the perikaryon of a JE-virus-infected neuron, 6 days p.i. The cystically dilated cisternae are filled with ER vesicles *(arrowhead)* and virions *(arrow)*. The cytosolic surfaces of limiting RER membranes are sparsely lined by ribosomes. Note that many virions show the bull's-eye form. *N*, nucleus. × 72000; *bar* = 100 nm

infected PC12 cells, in which nascent RER is formed by the rows of ribosomes that surround membrane-unbounded cisternae (Hase 1993). It appears, therefore, that the formation of new RER by rows of ribosomes surrounding membrane-unbounded cisternae seems to be the main motif of the RER morphogenesis in host cells in association with JE virus replication. The hypertrophic RER probably took shape through the formation of reticular RER and showed large areas of the cisternal surfaces where RER membranes were absent. It has been shown that the rows of ribosomes representing the vgRNA-polysomes synthesize the viral proteins at the membrane-unbounded cisternal surface of the reticular RER in JE-virus-infected PC12 cells (Hase 1993). Therefore, the fuzzy zone observed at the membraneless cisternal surface of the hypertrophic RER in infected neurons might represent viral membrane proteins synthesized on the rows of ribosomes together with lipids that accumulated at the interface between the cisterna and the cytosol. Eventually, RER membrane seemed to assemble at the interface. In the later phase of infection, the hypertrophic RER degenerated into the cystic RER whose dilated cisternae were packed with viral products.

With the acceptance of the signal hypothesis (Blobel and Dobberstein 1975), it is generally believed that RER morphogenesis occurs by the docking of mRNA-polysomes onto the cisternal membrane system of the existing endoplasmic reticulum (ER) (docking hypothesis). However, as in virally infected PC12 cells (Hase 1993), the present observations in the virally infected neurons demonstrated no cisternal membrane system, onto which polysomes were supposed to dock, prior to and during

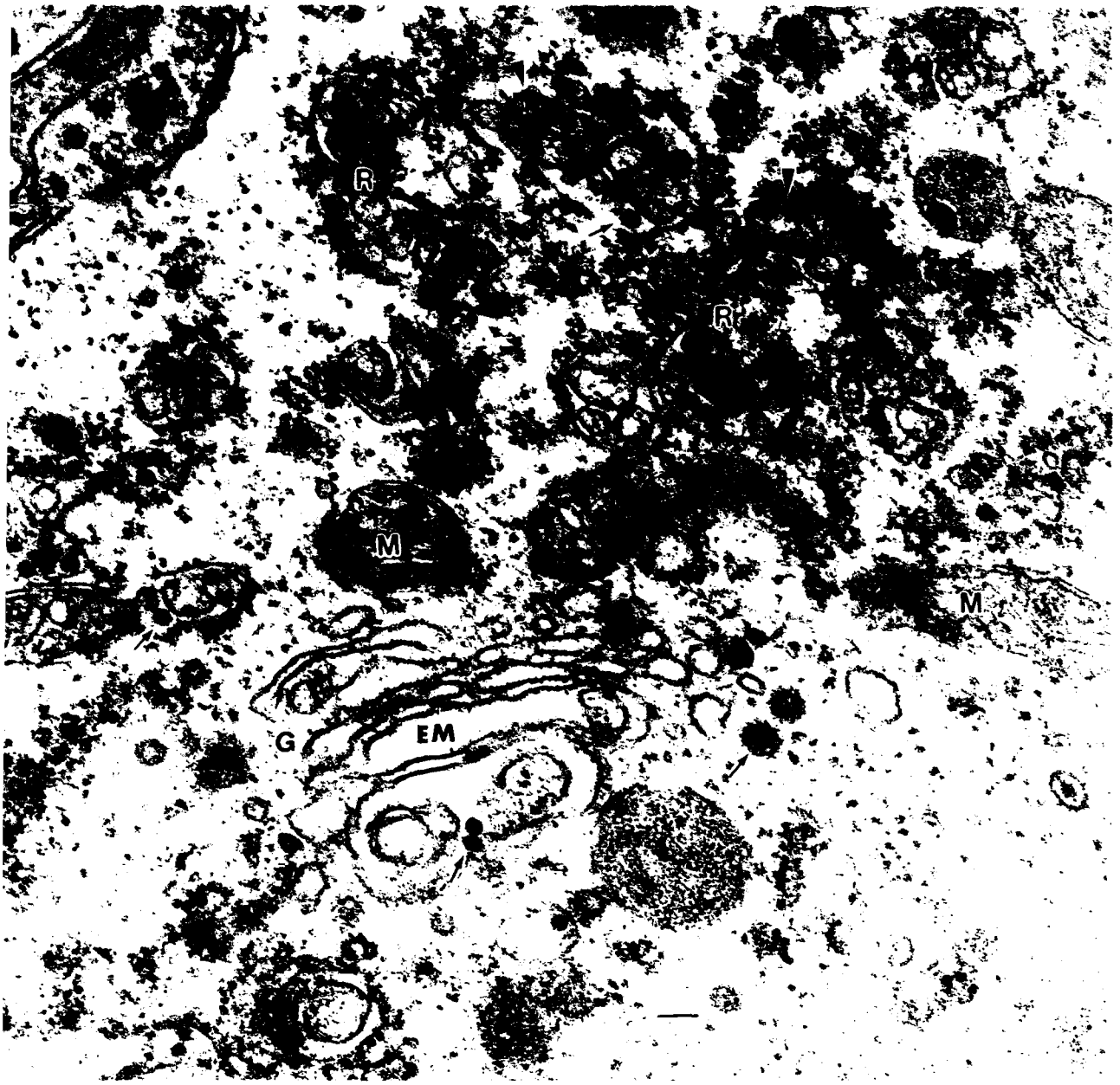

Fig. 8. A JE-virus-infected neuron in the cerebral cortex, 6 days p.i. RER (*R*) shows cystic degeneration, with cystically dilated cisternae being filled with ER vesicles (*arrowhead*) and virions (*arrow*). A small, localized Golgi complex (*G*) shows dilations of its saccules containing the electron-lucent material (*EM*). Virions (*arrow*) are also seen within coated vesicles, indicating that the Golgi complex is still releasing virions within coated vesicles into the cytoplasm for secretion. × 66000; *bar* = 100 nm

he RER morphogenesis. Therefore, it seems that the RER morphogenesis in JE-virus-infected cells does not occur simply by the docking of polysomes onto the existing ER membrane but that the nascent RER emerges through the formation of rows of ribosomes by polysomes surrounding membrane-unbounded cisternae. RER membranes apparently assemble subsequently at the membraneless cisternal surfaces during the RER morphogenesis. Probably reflecting these happenings, the hypertrophic RER in infected neurons displayed two morphologically characteristic features: (a) large areas of cisternal surfaces lacking limiting RER membranes and (b) diffusely dilated RER cisternae containing virions and ER vesicles.

Structural damage to the Golgi apparatus consisting of the fragmentation and dissolution of Golgi membranes and the accumulation of the electron-lucent material occurred consistently throughout the viral replication in neurons. In the early phase of infection, extensive damage occurred in the fine membrane network of the Golgi apparatus seen in normal neurons, raising the suspicion that some viral proteins synthesized at RER and transported to the Golgi apparatus were responsible for the damage. The nucleotide sequence of the vgRNA

of JE virus has been completely known (McAda et al. 1987; Sumiyoshi et al. 1987; Nitayaphan et al. 1990). The vgRNA has a single, long reading frame which translates the viral polyprotein, consisting of 10 vira. proteins, C-prM-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5. C-prM-E are the viral structural proteins which are assembled into virions in the RER cisterna. The assembled virions are shown to be transported to the Golgi apparatus and to pass through the Golgi apparatus for secretion without causing any recognizable structural damage to the organelle (Hase et al. 1987, 1990a). Among the nonstructural proteins, NS1 is a glycosylated protein that is known to be secreted extracellularly (Mason 1989). NS2a, 2b and NS4a, 4b are hydrophobic proteins on the basis of their corresponding nucleotide sequences and probably associate themselves with membrane. NS3 and NS5 are relatively large, non-glycosylated hydrophilic proteins; NS5 is thought to be the viral RNA-dependent RNA polymerase, and NS3 is thought to be a polymerase-associated protein (Rice et al. 1985). Since the flavivirus-encoded proteins are produced by processing of a single polyprotein (Rice et al. 1985), the flavivirus replication produces large excess of nonstructural proteins in the cytoplasm of host cells as the viral structural proteins are assembled into virions and released extracellularly. Little is known at present about the fates of the nonstructural proteins except NS1 which is glycosylated and known to be secreted extracellularly (Mason 1989). Therefore, there is a possibility that some of the nonstructural proteins are transported to the Golgi apparatus and remain there unprocessed because they are not secretory in nature. In this regard, the electron-lucent material observed in the Golgi apparatus during the viral replication in this study might represent viral proteins which accumulated in the Golgi apparatus.

On the basis of the present morphological observations, it seems reasonable to conceive the following sequence of events as the pathogenic mechanism involved in the infection of neurons with JE virus. (a) Upon entering neurons, the vgRNA replicates vigorously in the perikaryon and produces abundant vgRNA-polysomes. (b) The vgRNA-polysomes replace cellular mRNA-polysomes at RER and translate the viral polyprotein, from which individual viral proteins are enzymatically cleaved. (c) The viral proteins are discharged into the RER cisterna for the viral assembly, and the assembled virions and other viral proteins in the cisterna are transported to the Golgi apparatus. (d) The virions are processed at the Golgi apparatus and secreted extracellularly; on the other hand, some viral proteins apparently can not be processed at the Golgi apparatus and accumulate in and cause functional derangement and structural damage to the organelle. (e) The progressive damage to the Golgi apparatus results in impairment of secretory flow and degeneration of the secretory system with resultant cessation of secretion and membrane turnover in infected neurons. Therefore, JE virus apparently replicates successfully in the host secretory system including RER and the Golgi apparatus until the secretory system itself eventually breaks down as the result of accumulation of some noxious viral products. In this respect, it is not only of academic interest but also of practical significance to see that an exogenous mRNA such as the vgRNA of JE virus, introduced directly into the cytoplasm of host cells, functions at the host secretory system, with resultant secretion by the cells of the foreign proteins encoded in the exogenous mRNA.

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